

ORIGINAL ARTICLE

Transfusion-associated TT virus co-infection in patients with hepatitis C virus is associated with type II mixed cryoglobulinemia but not with B-cell non-Hodgkin lymphoma

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Objective To assess the prevalence of TT virus (TTV) infection in a series of patients with chronic hepatitis C virus (HCV) infection, with or without benign (mixed cryoglobulinemia) or malignant (B-cell non-Hodgkin lymphoma (B-NHL)) lymphoproliferative disease.

Methods Sixty-six HCV patients were studied, including patients with mixed cryoglobulinemia ($n=30$), B-NHL ($n=15$), and no mixed cryoglobulinemia or B-NHL ($n=21$). All HCV patients had increased transaminase levels and were HCV RNA positive. Patients were considered to have mixed cryoglobulinemia if two successive determinations of their serum cryoglobulin level were above 0.05 g/L. Mixed cryoglobulinemia-negative patients never had mixed cryoglobulins in their serum on multiple determinations. Subjects without HCV infection included 79 patients with histologically proven B-NHL, and 50 healthy blood donors. Serum samples were analyzed for TTV DNA by nested polymerase chain reaction, with two couples of primers in different regions of the genome, in two independent laboratories.

Results In the group of HCV-positive patients, TTV DNA was found in one of 15 (6.7%) patients with B-NHL, and in nine of 51 (17.6%, $P=0.43$) of those without B-NHL. Among HCV-positive patients without B-NHL, TTV DNA was more frequently found in those with type II mixed cryoglobulinemia vasculitis than in those without it (six of 16 (37.5%) versus two of 21 (9.5%), $P=0.05$). In subjects without HCV infection, TTV DNA was present in 10 of 79 (12.7%) patients with B-NHL and in seven of 50 (14.0%, $P=0.82$) blood donors.

Conclusion In patients chronically infected with HCV, TTV co-infection: (1) is not associated with the presence of B-NHL; and (2) is more frequently found in patients presenting a type II mixed cryoglobulinemia vasculitis.

Keywords Hepatitis C virus, mixed cryoglobulinemia, lymphoproliferative disease, lymphoma, B-cell non-Hodgkin lymphoma, transfusion-associated virus, TTV

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The most frequent extrahepatic manifestation associated with hepatitis C virus (HCV) infection

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is type II mixed cryoglobulinemia, which is a low-grade lymphoproliferative disease, found in 40–80% of cases [1–6]. Recent studies have suggested that other malignant lymphoproliferative diseases may be associated with chronic HCV infection [7–10]. A particularly high rate of HCV infection has been found in patients with B-cell non-Hodgkin lymphoma (B-NHL) [11,12]. Factors associated

with mixed cryoglobulinemia or B-NHL in patients with HCV chronic infection remain unknown.

TT virus (TTV) is a single-stranded DNA virus recently cloned from a patient with post-transfusion hepatitis [13]. This virus belongs to a new virus family designated Circinoviridae [14]. The analysis of its genetic organization reveals that the virus has a genome of approximately 3800 nucleotides. The TTV genome includes at least two hypothetical open reading frames (ORF1 and ORF2), composed, respectively, of 770 and 202 nucleotides, and several genotypes of TTV have been reported [13–15]. TTV DNA has been found to be associated with various liver diseases, including cryptogenetic cirrhosis, fulminant hepatitis [16–19] and hepatocellular carcinoma [20,21], but it is still unknown whether TTV can cause liver or non-liver disease in humans [22,23]. A high prevalence of TTV infection has been reported in bone marrow transplant recipients, with possible replication in hematopoietic cells [24].

To investigate a possible role of TTV in extra-hepatic immunologic disorders associated with HCV chronic infection, we performed a study to assess the prevalence of TTV DNA in a series of patients with chronic HCV infection with or without benign (mixed cryoglobulinemia) or malignant (B-NHL) lymphoproliferative disease.

PATIENTS

We studied different groups of consecutive patients with or without chronic HCV infection, and with or without B-NHL, from the Internal Medicine Department or Hematology Department of our university hospital.

Fifty-one patients (21 males, 30 females, mean age 55 years (range 18–81)) had chronic HCV infection without NHL. Chronic HCV infection was defined by alanine aminotransferase levels of more than twice the upper limit of the normal range, anti-HCV antibodies detected by third-generation tests (ELISA), liver biopsy findings compatible with chronic hepatitis C, and no other cause of liver dysfunction (e.g. chronic hepatitis B, autoimmune hepatitis or primary biliary cirrhosis). HCV-mixed cryoglobulinemia-associated vasculitis was noted in 16 out of 51 patients who had type II mixed cryoglobulinemia and at least one of the following clinical features: peripheral neuropathy, glomerulonephritis, skin purpura, or cerebral vasculitis. Fourteen HCV

patients had type III cryoglobulins without any associated extrahepatic manifestation, particularly without vasculitis. Twenty-one HCV-positive patients never had cryoglobulinemia on multiple determinations.

Ninety-four patients (30 males, 64 females, mean age 58 years (range 23–78)) had B-NHL, with ($n = 15$) or without ($n = 79$) chronic HCV infection. Lymphoma was diagnosed on the basis of morphologic evaluation of lymph node or extranodal tissue, including bone marrow. B-NHL was histologically classified using the Revised European-American Lymphoma (REAL) classification [25].

In addition, we studied a control group including 50 healthy blood donors (19 males, 31 females, mean age 51 years (range 22–67)) with normal levels of serum alanine aminotransferase, and without anti-HCV antibodies, cryoglobulinemia or lymphoma.

No subjects in either group were HIV positive.

METHODS

HCV serum markers

The serologic status of patients was determined using two specific third-generation immunoassays, a microparticle enzyme immunoassay (AxSYM HCV version 3.0, Abbott, Les Ulis, France) and an indirect immunoenzymatic technique (Monolisa anti-HCV Plus, Sanofi Diagnostic Pasteur, Marne la Coquette, France) performed according to the manufacturers' instructions. A patient was defined as positive when the two tests were positive according to each test specification. Sera used to detect HCV RNA were stored at -80°C and not thawed prior to use, to avoid false negativity due to RNA destruction by RNases and false positivity due to contamination [26]. Serum RNA was extracted, reverse transcribed to make cDNA, and amplified by PCR as previously described [27]. HCV genotyping was performed using a second-generation Line Probe Assay (LiPA, Innogenetics, Brussels, Belgium), which identifies the HCV main types and subtypes. All sera were negative for anti-HIV antibodies using commercial immunoassays (Abbott Laboratories, Diagnostic Pasteur, Paris, France).

Detection and characterization of cryoglobulins

Cryoglobulins were isolated from the patients' sera, purified, and characterized by immunoblotting at

37 °C as previously described [28]. In the present study, patients were considered to have a significant cryoglobulinemia if they had a minimum serum cryoglobulin level of 0.05 g/L in two determinations. Following the system of Brouet *et al.* [29], patients had either type II or type III mixed cryoglobulins, characterized, respectively, by the presence of a monoclonal or polyclonal rheumatoid factor component.

TTV DNA determination

All serum samples were analyzed for TTV DNA by nested polymerase chain reaction (PCR) in two independent laboratories in Marseille, France (Laboratory of Biochemistry, Hôpital La Conception, and Alfabio Laboratory). These two laboratories were selected for their ability to detect TTV from the 17 French laboratories specialized in molecular biology which belong to the GEMHEP group (the French Group of Molecular Study of Viral Hepatitis) [30]. TTV DNA detection was performed by PCR using two methods. The first method was based on an in-house PCR with the fully nested primers described by Simmonds *et al.* [15]. The second method, chosen by the GEMHEP group, was based on a hemi-nested PCR with degenerated primers derived from those described by Okamoto *et al.* [in 30]. A panel of controls prepared by an external investigator included 12 frozen samples: seven positive controls (obtained from five blood donors and two patients under hemodialysis), and five negative controls.

Liver biopsy

Histologic examination of liver biopsies included qualitative and quantitative analyses of the inflam-

matory activity and severity of fibrosis, which were classified using Knodell's score.

Statistical analysis

Statistical analysis used chi-square or Fisher's exact test for comparisons of percentages. Mean quantitative values were compared using the unpaired Student's *t*-test. Non-parametric analyses were done with the Mann-Whitney *U*-test. A Wilcoxon rank sum test was computed for ordinal variables. An assessment of the characteristics of HCV infection (age, gender, genotype, liver fibrosis) associated with TTV DNA positivity was performed using univariate and multivariate (logistic regression) analyses. All calculated *P*-values are two-tailed. Significance was assessed at *P* = 0.05.

RESULTS

In the group of HCV-positive patients, TTV DNA was found in one of 15 (6.7%) patients with B-NHL and nine of 51 (17.6%) of those without B-NHL [Table 1]. Among HCV-positive patients without B-NHL, TTV DNA was more frequently found in those with, compared to those without, type II mixed cryoglobulinemia vasculitis (six of 16 (37.5%) versus two of 21 (9.5%), *P* = 0.05). This difference was not associated with greater exposure to blood transfusion or intravenous drug abuse. The modes of acquiring HCV infection in patients with or without type II mixed cryoglobulinemia vasculitis were not different, i.e. transfusion (10/16 versus 12/21), intravenous drug abuse (3/16 versus 6/21), and unknown (3/16 versus 3/21). No factor tested showed a significant difference between TTV DNA-positive or -negative patients, i.e. age, gender, HCV genotype, liver

Table 1 Prevalence of serum TTV DNA in a series of patients with chronic HCV infection, with or without benign (mixed cryoglobulinemia) or malignant (B-NHL) lymphoproliferative disease

	TTV positive (number)	Number of patients	TTV positive (%)
HCV type II mixed cryoglobulinemia	6	16	37.5
HCV type III cryoglobulin	1	14	7.1
HCV positive, cryoglobulin-negative, without B-NHL	2	21	9.5
HCV positive with B-NHL	1	15	6.7
HCV negative with B-NHL	10	79	12.7
Healthy blood donors ^a	7	50	14.0

HCV, hepatitis C virus; B-NHL, B-cell non Hodgkin lymphoma.

^aHCV-negative without B-NHL.

fibrosis. In subjects without HCV infection, TTV DNA was present in 10 of 79 (12.7%) patients with B-NHL and seven of 50 (14.0%, $P=0.82$) blood donors. TTV DNA was found with a 100% concordance with both PCR methods in both independent laboratories.

DISCUSSION

Benign (mixed cryoglobulinemia) or malignant (B-NHL) lymphoproliferative disease has been frequently reported in association with chronic HCV infection [4–12]; the factors associated with such lymphoproliferative diseases in patients with HCV chronic infection remain unknown. TTV is a single-stranded DNA virus recently cloned from a patient with post-transfusion hepatitis [13]. TTV has been thought to be a new hepatitis virus, but to date it is still unknown whether TTV are responsible for liver disease or other diseases in humans [22,23].

The prevalence of TTV DNA in European and North American patients with chronic HCV infection has been reported to range from 12.5% [31,32] to 27% [33]. In the present study, we found a 17.6% prevalence of TTV DNA in French HCV-positive patients, which in agreement with these studies. We did not find a correlation between age, sex, HCV genotype, liver fibrosis, and TTV DNA positivity [34,35]. The prevalence of TTV DNA in patients with B-NHL was low and not different from that in blood donors, whether they had (6.7%) or did not have (12.7%) chronic HCV infection. The mechanism(s) that may promote B-NHL in HCV patients remains to be determined.

More interestingly, we found a higher prevalence of TTV DNA in HCV patients with type II mixed cryoglobulinemia vasculitis compared to those without cryoglobulinemia. This difference could not be attributed to a higher exposure to blood transfusion, intravenous drug abuse, or other confounding factors such as frequent hospital admissions, parenteral exposures, or plasmapheresis, none of which were statistically different in these populations. Previous studies searching for factors that may promote mixed cryoglobulin production, whether viral (HCV genotype, HCV viremia) [36–39], immunologic (immunoglobulin or lymphocyte subsets) [27,40], or infectious (hepatitis G virus, parvovirus B19) [41,42], were negative.

One possible mechanism for the development of vasculitis in HCV patients with mixed cryoglobu-

linemia suggested by Agnello and Abel [43] is that a small, high-molecular-weight small portion of the cryoglobulins initiate activation of endothelial cells, leading to altered vascular permeability, neutrophil infiltration, and vessel damage. An alternative mechanism involves hepatitis C virus-induced endothelial damage, as suggested by the finding of HCV antigen in normal vascular endothelial cells [44], anti-endothelial cell antibody-induced apoptosis [45], and excessive production of anti-endothelial cell antibodies in HCV cryoglobulinemia vasculitis [46]. TTV co-infection may then increase endothelial cell alterations, leading to more frequent mixed cryoglobulinemia vasculitis in HCV patients. The results, when testing for TTV DNA, are highly dependent on the primers chosen to detect the virus [47,48]. It is possible that the primers used in the present study underestimated the prevalence of TTV infection.

CONCLUSION

In patients chronically infected with HCV, TTV coinfection [1] is not associated with the presence of B-NHL [2], and is more frequently found in patients with type II mixed cryoglobulinemia vasculitis.

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